

# An Empirical Approach for Estimating the Equivalent Chain Length of Fatty Acid Methyl Esters in Multistep Temperature-Programmed Gas Chromatography

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## Abstract

The equivalent chain length (ECL) has been widely accepted as an identification tool for fatty acid methyl esters (FAMES). In this study, FAMES from the seed oil of Chinese mustard (*Brassica juncea*) analyzed on a 0.25-mm-i.d. capillary column coated with an SA-WAX are used as an example for the calculation of the ECL in single-, two-, three-, and four-step temperature-programmed gas chromatography. The ECLs of unsaturated FAMES tend to increase with temperature, and they agree well with those reported in the literature.

## Introduction

The retention index (I) system proposed by Kováts (1) has been widely accepted as a tentative general identification for organic compounds, and the equivalent chain length (ECL) (2,3) has been used specifically for the identification of fatty acid methyl esters (FAMES). The retention index and ECL are closely related and interconvertible (4). However, both the retention index and ECL are generally determined under isothermal conditions. Today, more and more complex mixtures are being analyzed, and it is not possible to resolve all FAMES in a single isothermal run or it is too tedious in normal practice. Therefore, temperature-programmed gas chromatography (TPGC) is more convenient. In addition, peak separation in TPGC is improved and peaks are narrower and elute in a shorter time (5).

Methods for the calculation of temperature-programmed retention indices and the ECL have been described in several studies (5–14). A comparative study of these methods was reported by Garcia Dominguez and Santinuste (14). The equation proposed by Curvers et al. (15) using thermodynamic

parameters seemed to produce the least error (14). Curvers' approach has been widely utilized and extended to cover multistep TPGC (16). Recently, Kittiratanapaiboon et al. (17) proposed to use equation 2, the combination of equation 1 proposed by Krisnangkura et al. (18) with the column slicing method of Cavalli and Guinchart (11), to forecast the retention times of FAMES in TPGC:

$$\ln k' = a + bn + \frac{c}{T} + \frac{dn}{T} \quad \text{Eq. 1}$$

where  $k'$  is the retention factor;  $T$  is the oven temperature (Kelvin);  $n$  is the carbon number or ECL; and  $a$ ,  $b$ ,  $c$ , and  $d$  are thermodynamically related column constants.

$$t_R = \frac{\sum_{i=1}^m t_m [1 + g (\theta_i + T_i)]}{m} \left( 1 + e^{(a + bn + \frac{c}{q_i} + \frac{dn}{q_i})} \right) \quad \text{Eq. 2}$$

where  $m$  is the number of elements,  $g$  is the temperature gradient;  $T_i$  is the initial temperature, and  $\theta_i$  is the temperature of the  $i$ th elements. In normal practice, the retention time directly obtained from the chromatogram is used for the identification of an unknown.

In this study, a program in Qbasic written by Cavalli and Guinchart (11) was modified to calculate the ECL of FAMES by using equation 2 in multistep TPGC.

## Experimental

### Materials

FAMES and  $n$ -paraffins were purchased from Sigma Chemical Co. (St. Louis, MO). Chinese mustard seed (*Brassica juncea*) was obtained from a grower's shop. Transmethylation of plant seed oils was carried out in situ with acid catalysis as described by Kalayasiri et al. (19) with methyl heptadecanoate as an internal standard.

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CLS
PRINT "Estimation of ECL of FAMES in Multi-step TPGC"
a = -9.134
b = -.5443
c = 1374.296
d = 405.091
n = 1000
DO
DO
INPUT "HOW MANY STAGE DO YOU HAVE IN YOUR TEMPERATURE PROGRAM?" ,r$
s = VAL (r$)
LOOP UNTIL s > 0
REDIM Ti (s) , ht (s) , Tf (s) , g (s)
INPUT "ENTER INITIAL TEMPERATURE OF STAGE No1" ; r$
Ti (1) = VAL (r$)
FOR i = 1 TO s
DO
INPUT "ENTER FINAL TEMPERATURE OF STAGE No"; i ; ":"; r$
Tf (i) = VAL (r$)
LOOP UNTIL Tf (i) > Ti (i)
IF i < s THEN Ti (i+1) = Tf (i)
DO
INPUT "HOLD TIME OF STAGE" ; i ; r$
ht (i) = VAL (r$)
LOOP UNTIL ht (i) >= 0
DO
INPUT "TEMPERATURE GRADIENT OF STAGE No"; i ; ":"; r$
g (i) = VAL (r$)
LOOP UNTIL g (i) > 0
NEXT i
PRINT "Ti" , "HOLD" , "Tf" , "G"
FOR i = 1 TO s
PRINT Ti (i) , ht (i) , Tf (i) , g (i)
NEXT i
INPUT "ARE THOSE VALUES CORRECT ? (Y/N) " , r$
LOOP WHILE (r$ <> "Y" AND r$ <> "y")
DO
DO
INPUT "ENTER THE VALUES OF DEAD TIME:" , r$
t0 = VAL (r$)
LOOP UNTIL t0 > 0
PRINT "DEAD TIME = " ; t0
INPUT "IS THE VALUE CORRECT ? (y/n) " , r$
LOOP WHILE (r$ <> "y" AND r$ <> "Y")
REDIM t (s, 1)
t(0, 1) = 0
FOR i = 1 TO s
t(i, 0) = t(i - 1, 1) + ht (i)
t(i, 1) = t(i, 0) + (Tf (i) - Ti (i)) / g (i)
NEXT i
DO
cn = 8
DO
INPUT "Enter retention time of peak you want to identify:" , r$
Rt = VAL (r$)
INPUT "IS THE VALUE CORRECT ? (y/n) " , r$
LOOP WHILE (r$ <> "y" AND r$ <> "Y")
FOR P = 0 TO 6
H = 10 ^ P
H = 1 / H
CN = CN - H
DO
cn = cn + H
x = a + b * cn
y = c + d * cn
tr = 0
FOR i = 0 TO n
'CALCULATION OF THE OVEN TEMPERATURE
j = 0
DO
j = j + 1
LOOP UNTIL tr < t (j, 1) OR j = s
IF tr < t (j, 0) THEN TEMP = Ti (j) ELSE TEMP = Ti (j) + g (j) * (tr - t (j, 0))
IF tr > t (s, 1) THEN TEMP = Tf (s)
tm = t0 + .0013 * (TEMP - Ti (1))
'CALCULATION OF tr+Dtr
tr = tr + tm * (1 + EXP (x + y / (TEMP + 273.15))) / n
NEXT i
DIF = tr - Rt
PRINT cn
LOOP UNTIL DIF > 0
IF ABS (DIF) < .001 THEN PRINT tr
cn = cn - H
NEXT p
PRINT "retention time = " ; tr ,
PRINT "Temperature = " ; TEMP ,
PRINT "ECL = " ; cn
INPUT "DO YOU WANT TO CALCULATE OTHER PEAK ? " , a$
LOOP WHILE a$ = "Y" OR a$ = "y"
END

```

**Figure 1.** A program in Qbasic (modified from that of Cavalli and Guinchar) for the calculation of the ECL.

## Gas chromatography

Gas chromatography (GC) analysis was performed on a Shimadzu (Kyoto, Japan) Model 14A. The instrument was equipped with a flame ionization detector, split/splitless injector, a C-R4A data processor (Shimadzu), and a polyethylene glycol (SA-WAX, Sigma-Aldrich Co., St. Louis, MO) capillary column (30-m  $\times$  0.25-mm i.d., 0.25- $\mu$ m film thickness). The carrier gas was nitrogen at the flow rate of 0.7–1.5 mL/min. Injector and detector temperatures were set at 250°C. In order to get a consistent injection time, a remote start for the data processor was attached to the syringe handle.

## Determination of the hold-up time

The secondary GC hold-up time ( $t_{ms}$ ) was calculated according to Watanachaiyong et al. (20). A series of C<sub>10</sub>–C<sub>15</sub> *n*-paraffins was chromatographed at 120–160°C at 5°C intervals. The retention times of *n*-paraffins at each temperature were used to calculate the hold-up times ( $t_m$ ) as described by Guardino et al. (21). The calculated  $t_m$  values and the retention times of *n*-paraffins were then used to establish the four thermodynamically related column constants (a, b, c, and d) for *n*-paraffins according to Krisnangkura et al. (18). The numerical values of the four constants are a = –6.39, b = –0.68, c = –1017.88, and d = 477.08. Equation 3 was derived from the substitution of these four constants into equation 1 and then rearranged. It is utilized for the calculation of  $t_{ms}$  (20) from the retention time of hexane (the solvent used for the sample).

$$t_m = t_R \left( 1 + e^{\left( -6.39 - 0.68n - \frac{1017.88}{1} + \frac{477.08n}{1} \right)} \right)^{-1} \quad \text{Eq. 3}$$

## Column constants for FAME

The four column constants of equation 1 for FAME were determined as described by Krisnangkura et al. (18). The secondary hold-up time was used in place of the hexane retention time. The numerical values for the four column constants were a = –9.134, b = –0.5443, c = 1374.296, and d = 405.091. Equation 4 was derived by substituting these four column constants into equation 2 and then used for the calculation of the ECL in TPGC. The calculation was carried out on a personal computer with a program written in Qbasic (Figure 1).

$$t_R = \frac{\sum_{i=1}^m t_m [1 + g(\theta_i + T_i)]}{m} \left( 1 + e^{\left( -9.134 - 0.5443n + \frac{1374.296}{T_i} + \frac{405.091n}{T_i} \right)} \right) \quad \text{Eq. 4}$$

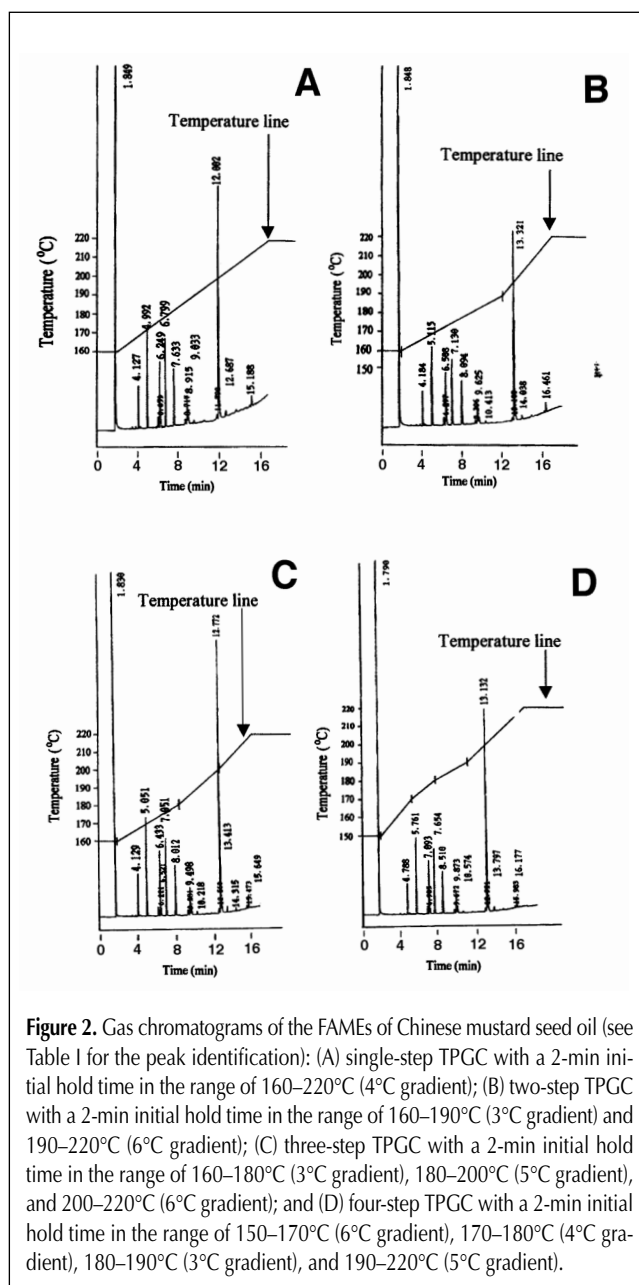
## Qbasic program for the calculation of $t_R$

The Qbasic program used was slightly modified from that previously described by Cavalli and Guinchard (11). The calculation could be started at any ECL value, but in this illustration an ECL of 8 was used at the start. The calculated retention time ( $t_{R(cal)}$ ) was then compared with the retention

time of the unknown peak ( $t_{R(exp)}$ ). If  $t_{R(cal)}$  was less than  $t_{R(exp)}$ , then a  $1/10^p$  value (whenever  $p = 0$  to 4) was added. For  $p = 0$ , the ECL was increased by 1 unit at a time until the  $t_{R(cal)}$  was greater than  $t_{R(exp)}$ , suggesting that the assigned ECL was too high. The program then exited the calculation loop. If the absolute difference between  $t_{R(cal)}$  and  $t_{R(exp)}$  was less than 0.001, the ECL was printed. However, if the absolute difference between  $t_{R(cal)}$  and  $t_{R(exp)}$  was greater than 0.001, the value of  $1/10^p$  was taken out of the ECL. After assigning the next  $p$  value, calculation in the loop was resumed.

## Results and Discussion

The seed oil of Chinese mustard contains polyunsaturated and long chain fatty acids (19), thus its FAMES were suitable as



**Figure 2.** Gas chromatograms of the FAMES of Chinese mustard seed oil (see Table I for the peak identification): (A) single-step TPGC with a 2-min initial hold time in the range of 160–220°C (4°C gradient); (B) two-step TPGC with a 2-min initial hold time in the range of 160–190°C (3°C gradient) and 190–220°C (6°C gradient); (C) three-step TPGC with a 2-min initial hold time in the range of 160–180°C (3°C gradient), 180–200°C (5°C gradient), and 200–220°C (6°C gradient); and (D) four-step TPGC with a 2-min initial hold time in the range of 150–170°C (6°C gradient), 170–180°C (4°C gradient), 180–190°C (3°C gradient), and 190–220°C (5°C gradient).

the demonstrating sample in this study. It was analyzed in one-, two-, three-, and four-step TPGC. The column temperature and temperature program rate were chosen such that the chromatographic peaks eluted from the column at every stage, as shown in Figure 2. Retention times were printed at the top of the peaks by the C-R4A data processor, and they were computed for the ECL with the Qbasic program (Figure 1). Results are summarized in Table I.

The calculated ECL values of the unsaturated FAMES were slightly different than those reported in the literature (18). The differences could arise either from the change in the ECL with temperature (5) or the liquid phase used in this study was not exactly the same as those reported in the literature. In order to clarify this discrepancy, the ECL values of the FAMES from Chinese mustard oil were analyzed isothermally between 160°C and 210°C. The results are summarized in Table II. It

can be seen that the ECLs calculated by using equation 1 agreed well with those calculated by the classical method of Miwa et al. (3). Also, both were very sensitive to temperature. The higher number of double bonds or longer chain length tended to yield higher ECL values at a higher temperature (Table II). Results from the isothermal study suggested that a slight change in the ECLs in the multistep TPGC was probably because of the effect of temperature. However, the exact ECL values in multistep temperature-programmed GC depends very much on the accurate determination of the equivalent temperature.

It may be concluded that the proposed method provides a convenient and accurate method for the identification of FAMES in TPGC without a FAME as a reference. Also, this method can be extended to other organic compounds when the retention index is used in place of the ECL.

**Table I. ECL for FAMES of Chinese Mustard Seed Calculated at Various Multistep TPGC**

Single step*		Two step†		Three step‡		Four step§		Tentative identification
$t_{R(hex)} = 1.849$ min	$t_M = 1.845$ min	$t_{R(hex)} = 1.848$ min	$t_M = 1.844$ min	$t_{R(hex)} = 1.830$ min	$t_M = 1.826$ min	$t_{R(hex)} = 1.790$ min	$t_M = 1.786$ min	
$t_R$ (min)	ECL	$t_R$ (min)	ECL	$t_R$ (min)	ECL	$t_R$ (min)	ECL	
4.127	16.01	4.184	16.03	4.129	16.01	4.788	16.01	16:0
4.992	17.00	5.115	17.02	5.051	17.00	5.761	17.00	17:0**
6.059	18.00	6.297	18.02	6.221	18.00	6.905	18.00	18:0
6.249	18.16	6.508	18.17	6.433	18.16	7.093	18.15	18:1 (n-9)
6.799	18.60	7.130	18.61	7.051	18.60	7.654	18.59	18:2 (n-6)
7.633	19.23	8.094	19.24	8.012	19.22	8.510	19.23	18:3 (n-3)
8.717	19.99	9.396	20.01	9.281	19.99	9.672	20.02	20:0
8.915	20.13	9.625	20.14	9.498	20.12	9.873	20.15	20:1 (n-9)
9.560††	20.56	10.413	20.58	10.218	20.55	10.574	20.60	20:2 (n-6)
11.792	22.01	13.103	22.02	12.560	21.99	12.921	22.05	22:0
12.002	22.15	13.321	22.14	12.772	22.13	13.132	22.18	22:1 (n-9)
15.188	24.20	16.461	24.18	15.649	24.18	16.177	24.23	24:1 (n-9)

\* 2-min initial hold time at 160–220°C (4°C/min).  
† 2-min initial hold time at 160–190°C (3°C/min) and 190–220°C (6°C/min).  
‡ 2-min initial hold time at 160–180°C (3°C/min), 180–200°C (5°C/min), and 200–220°C (6°C/min).  
§ 2-min initial hold time at 150–170°C (6°C/min), 170–180°C (4°C/min), 180–190°C (3°C/min), and 190–220°C (5°C/min).  
\*\* Internal standard.  
††  $t_R$  was not printed out in the chromatogram.

**Table II. Comparison of the ECL of Unsaturated FAMES from the Seed Oil of Chinese Mustard Calculated at Various Temperatures**

FAMES	160°C		180°C		190°C		210°C		$\Delta ECL^{*,\dagger}$	$\Delta ECL^{\ddagger,\S}$
	ECL*	ECL†	ECL*	ECL†	ECL*	ECL†	ECL*	ECL†		
18:1 (n-9)	18.14	18.17	18.20	18.23	18.22	18.23	18.26	18.27	0.024	0.020
18:2 (n-6)	18.57	18.60	18.65	18.69	18.69	18.71	18.77	18.79	0.040	0.038
18:3 (n-3)	19.17	19.21	19.25	19.33	19.34	19.37	19.45	19.47	0.056	0.052
20:1 (n-9)	20.10	20.15	20.15	20.20	20.18	20.21	20.24	20.26	0.028	0.022
22:1 (n-9)	22.06	22.13	22.13	22.19	22.16	22.21	22.21	22.26	0.030	0.026
22:2 (n-6)	22.44	22.51	22.53	22.61	22.59	22.64	22.68	22.73	0.048	0.044
24:1 (n-9)	nd <sup>§</sup>	nd <sup>§</sup>	24.08	24.17	24.12	24.19	24.19	24.26	0.037	0.030

\* Calculated by the classical method of Miwa et al. (3).  
† Calculated by equation 1.  
‡ Change in the ECL per 10°C.  
§ nd, the peak was very broad.

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